Award Number: **W81XWH-07-1-0022**

TITLE: **Enhancement of Vitamin D Action in Prostate Cancer through Silencing of CYP24**

PRINCIPAL INVESTIGATOR: **Dolores J. Lamb, Ph.D.**

CONTRACTING ORGANIZATION: **Baylor College of Medicine, Houston, TX, 77030**

REPORT DATE: **February, 2010**

TYPE OF REPORT: **Annual**

PREPARED FOR: **U.S. Army Medical Research and Materiel Command**
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*
01-02-2010

2. REPORT TYPE
Annual

3. DATES COVERED *(From - To)*
15 JAN 2008 - 14 JAN 2010

4. TITLE AND SUBTITLE
Enhancement of Vitamin D Action in Prostate Cancer through Silencing of CYP24

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0022

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dolores J. Lamb, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine

Houston, Texas 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
And Materiel Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This study focuses on the enzyme, CYP24 which hydroxylates vitamin D acting to catalyze the first step in the breakdown of Vitamin D, effectively limiting this growth inhibitory signaling pathway. We are testing the hypothesis that hrough the inhibition of CYP24 using an siRNA approach we can convert prostate cancer cells that are resistant to the antiproliferative actions of Vitamin D to cells that are growth inhibited at low concentrations of Vitamin D. Inhibition of 1,25(OH)2D3 CYP24 mediated metabolism to potentiate Vitamin D actions in prostate cancer shows great potential for both a chemopreventative approach and the treatment of advanced hormone refractory cancer in patients. We have tested CYP24 siRNA constructs, ketoconazole and silencer control siRNA on three cell lines (LNCaP, PC3 and DU145) and evaluated CYP24 protein expression, mRNA expression, and growth inhibition. We are in the process of developing the stable transfected cell lines and optimal approach to enhance Vitamin D action in resistant cells.

15. SUBJECT TERMS
Prostate cancer, vitamin D, CYP-24 (24-hydroxylase), growth inhibition, apoptosis

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

PROGRESS REPORT

Specific Aim #1 To enhance Vitamin D inhibition of prostate cancer growth through inhibition of CYP24.

The summary below describes our progress towards meeting all aims on our funded proposal.

C.1. CYP24A1is Over expressed In Human Prostate Cancer: It was important to first assess the mRNA levels of endogenous CYP24A1 in adenocarcinomas of the human prostate. cDNAs obtained from pathologist-verified human prostate biopsies representing different TNM (Tumor, Node and Metastasis) stages of prostate cancer as well as benign prostatic hyperplasia (BPH) were analyzed by quantitative PCR. Despite the variability observed between individuals, the benign tissues expressed low levels of CYP24A1 while the majority of the analyzed adenocarcinomas (73%) had CYP24A1 highly expressed (Figure 1A). When the relative levels of mRNA were examined as mean values by TNM stage, a marked elevation of CYP24A1 was

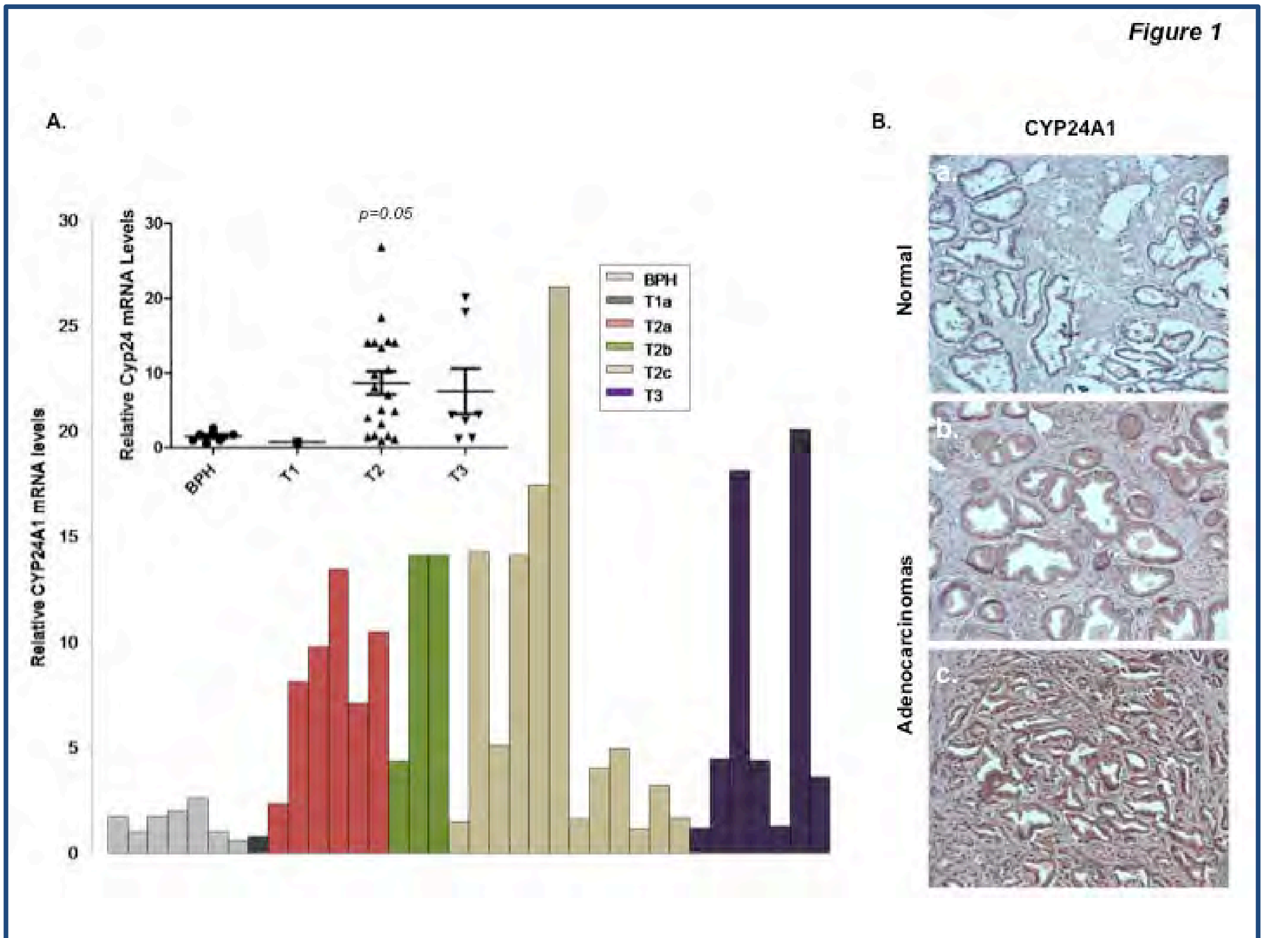


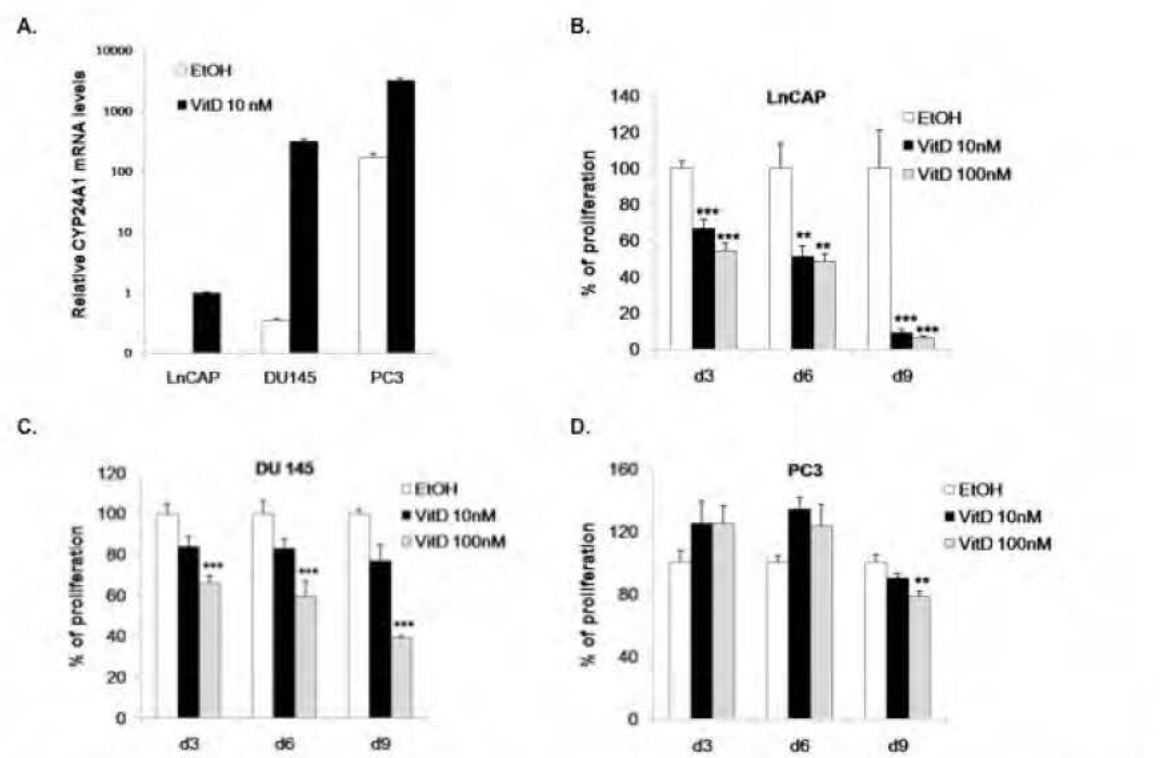
Figure 1: Over expression of CYP24A1 in men with prostate cancer. A. CYP24A1 gene expression levels across the stages of human prostate cancer progression. TaqMan QPCR was performed using specific CYP24A1 primers. β actin served as an internal control. Patients' cDNA were prepared from well-documented cancer biopsy samples, normalized and assembled into ready-to-use gene expression panels. Clinicopathological information for each patient was provided by the supplier (Origene, MD, USA). Data were analyzed using the comparative CT method with the values normalized to β actin levels. Figure in inset is a scatter plot presenting qPCR results as averages of relative CYP24A1 mRNA levels in individuals presenting identical TNM stage**. B.** Marked increase of cytoplasmic CYP24A1 immunostaining (brown) in representative human prostate adenocarcinomas compared to normal human prostate.

observed with increasing pathological grades of prostate cancer, with some heterogeneity at T3 (Figure 1A). Consistently, CYP24A1 protein was over expressed in adenocarcinomas of prostate compared to normal human prostate (Figure 1B). While normal cells demonstrated faint cellular staining for the catabolic enzyme, immunodetection of CYP24A1 revealed intense cytoplasmic staining in epithelial cells of both low and high Gleason grade adenocarcinomas (Figure 1B). Taken together, these data demonstrate for the first time, the changes of expression of CYP24A1 in patients with prostate cancer and highlight the potential key role of CYP24A1 in prostate cancer.

C.2. Basal Levels of CYP24A1 mRNA Correlate With The Growth Response To Vitamin D In Prostate Cancer Cells: Increased CYP24A1 expression in human prostate cancer tissues presumably decreases the intra-tumoral $1,25(OH)_2D_3$ levels effectively counteracting the anti-proliferative effects of calcitriol. Our data supports this hypothesis. Indeed, LnCAP, with the lowest expression levels of CYP24A1(Fig 2.a), is most responsive to the anti-proliferative effect of calcitriol (Fig 2.b). In contrast, PC3 displays the highest basal levels of CYP24A1and is the most resistant to the inhibitory effect of calcitriol on cell growth (Fig 2.b).

Figure 2: Inverse correlation between endogenous levels of CYP241 mRNA and growth abrogation action of vitamin D3. A. qRT-PCR analysis of endogenous CYP24A1 mRNA levels in presence of 10nM Vitamin D3 or vehicle (ethanol; EtOH) in LnCAP, DU145 and PC3 prostate cancer lines. GAPDH served as the internal control. **B-D**. Cell proliferation assays performed in presence of 10 nM or 100 nm calcitriol or ethanol after 3, 6 and 9 days of treatment in LnCAP (B), DU145 (C) and PC3 (D) (* if P< .05; ** if P< .01; *** if P< .001, ANOVA).

Figure 2

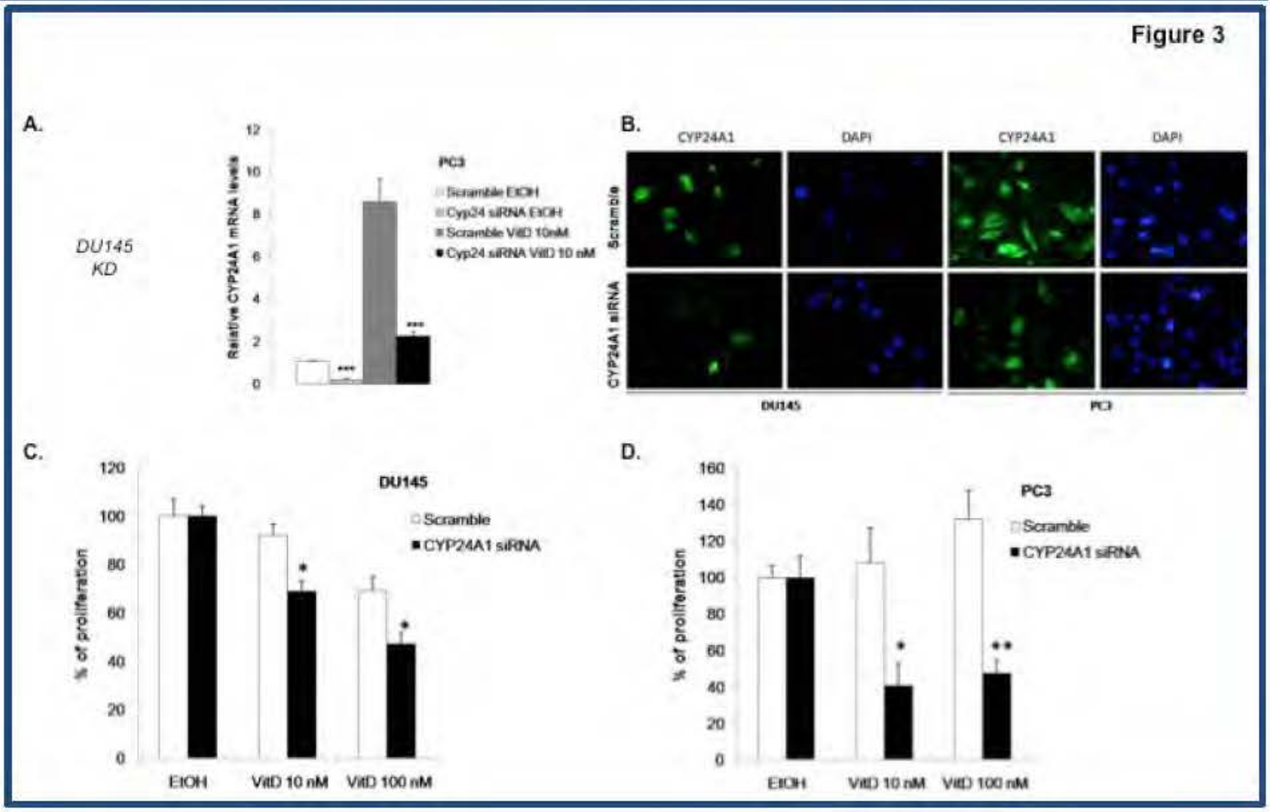


3. ***Optimal Transcript Design for Efficient CYP24A1Inhibition:*** We first assessed by qRT-PCR, the efficacy of inhibition of CYP24A1 expression. siRNA oligonucleotides were

synthesized by Dharmacon Research, In. (Lafayette, CO). Cells were transfected with CYP24A1siRNA oligos or scrambled siRNA. The optimal approach used CYP24A1 ON TARGETplus SMART pool at a concentration of 20-40 ng/ml (http://www.dharmacon.com/CatalogSearch/ConsolidatedSearch.aspx?searchTerm=cyp24a1&searchTarget=1591_+CYP24A1).

Using this approach, we were able to selectively inhibit CYP24A1expression in prostate cancer cell lines using RNA interference. The relative mRNA levels of CYP24A1 were efficiently knocked down by siRNA in absence or presence of vitamin D3 (**Figure 3A**). When compared to cells transfected with the non-targeting siRNA, the abrogation of expression by CYP24A1 siRNA in PC3 cells was about 80% in constitutive conditions and 74 % in presence of 10 nM vitamin D3 (**Figure 3A**). The knockdown of CYP24A1 mRNA translated into reduced levels of CYP24A1 protein, as reflected by the attenuation of the punctuated staining seen in cells transfected with CYP24A1 siRNA compared to control cells transfected with scramble (Figure 3B). To assess the cellular consequences of siRNA-mediated silencing of CYP24A1 gene expression, proliferation assays were performed. While PC3 and DU145 cells were not responsive to the growth inhibition mediated by vitamin D3 (Figures 2C and 2D), CYP24A1 siRNA significantly enhanced the anti-proliferative action of vitamin D3 (Figures 3C and 3D). In a cellular context

Figure 3: Effect of selective inhibition of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D3. A. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D3 or vehicle (EtOH) in DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non-targeting siRNA (Scramble). GAPDH served as the internal control. **B.** Immunofluorescent staining of CYP24A1 protein of DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non-targeting siRNA (Scramble) and incubated in presence of Vitamin D3 10 nM. DAPI was used for cell nuclei visualization. **C-D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol after 6 days of treatment in DU145 (**C**) and PC3 (**D**) transiently transfected with CYP24A1 siRNA or non targeting siRNA (Scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA. * if P< .05; ** if P< .01; *** if P< .001.



which is very responsive to vitamin D3 e.g. LnCAP, transient transfection of CYP24A1 siRNA also produced a significant enhancement of the growth inhibitory action of calcitriol

Figure 4

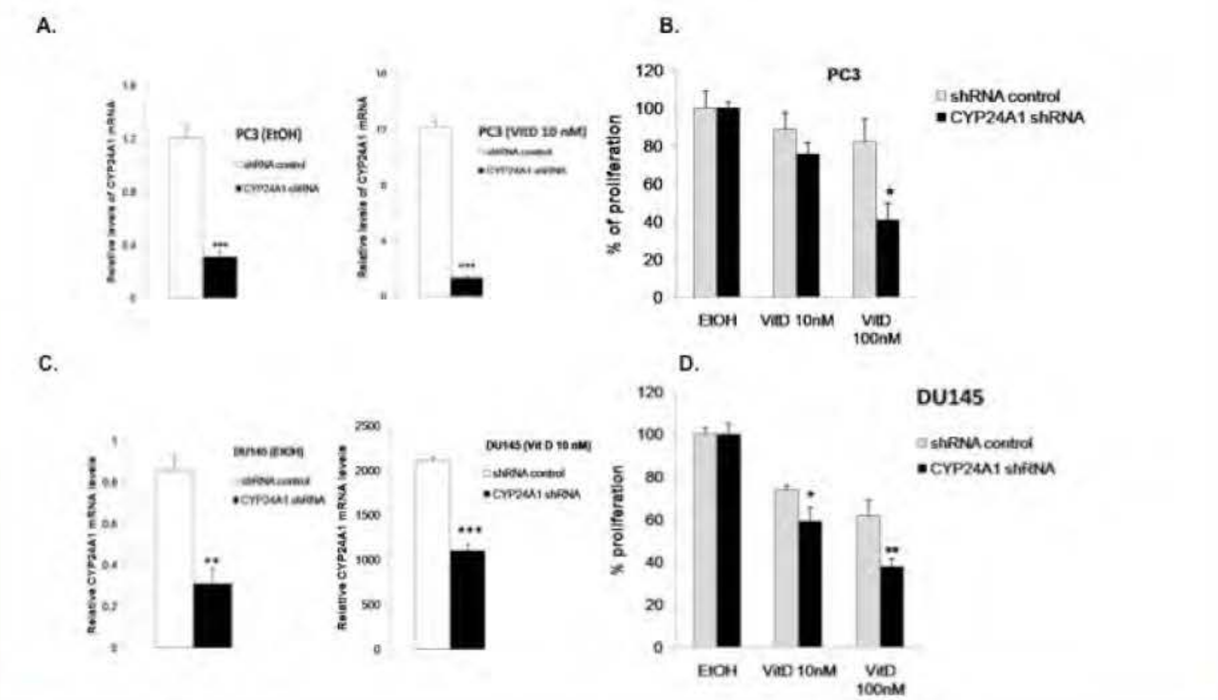


Figure 4: Generation of stable clones expressing shRNA and impact of sustained alteration of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D3. A and C. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D3 or vehicle (EtOH) in PC3 (**A**) and DU145 (**C**) stably transfected with CYP24A1 shRNA or a non-targeting shRNA (Scramble). GAPDH served as the internal control. B and D. Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol for 6 days in stable PC3 (**B**) and DU145 (**D**) expressing CYP24A1 shRNA or non targeting shRNA (Scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA, * if $P< .05$; ** if $P< .01$; *** if $P< .001$.

4. ***Selective Inhibition of CYP24A1 Enhances The Anti-Proliferative Effect of 1,25(OH)$_2$D$_3$ in Stably Transduced Prostate Cancer Cell Lines***: To assess the impact of a sustained expression of CYP24A1 siRNA on the growth abrogation mediated by vitamin D3, we generated LnCAP, DU145 and PC3 stable cell lines expressing CYP24A1shRNA or non-targeting shRNA. The random integration into the genome of the vector-based shRNA led to a significant knock down of the CYP24A1expression in presence or absence of calcitriol (**Figures 4A and 4C**). Indeed, when compared to cells with non-targeting shRNA, relative CYP24A1 mRNA levels were reduced by 75% in PC3 and 85% in DU145 in presence of ethanol, and the knockdown was about 90% for PC3 and 60% for DU145 in presence of vitamin D3. In these clonal conditions, PC3 showed an improvement of its growth response to vitamin D3, reaching significance at 100 nM (Figure 4B). In DU145, a significant reduction of cell proliferation was already achieved at 10 nM of vitamin D3 (Figure 4D). Hence, the prolonged abrogation of CYP24A1 gene expression enhanced the 1,25(OH)2D3-mediated growth inhibition in prostate cancer cells.

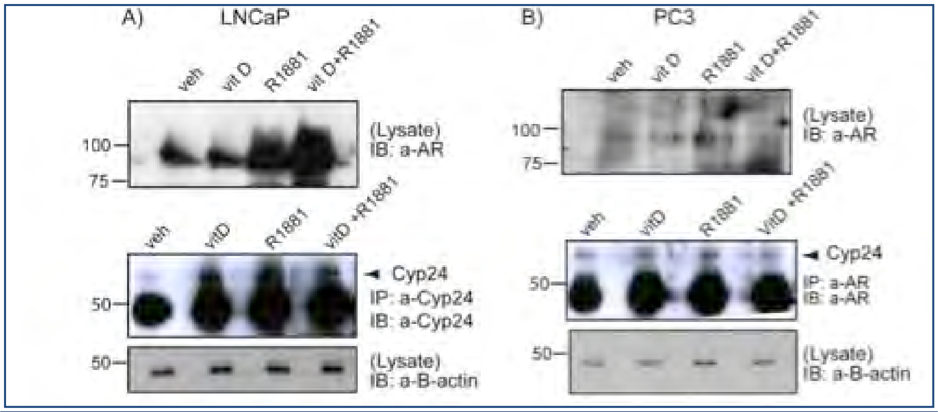
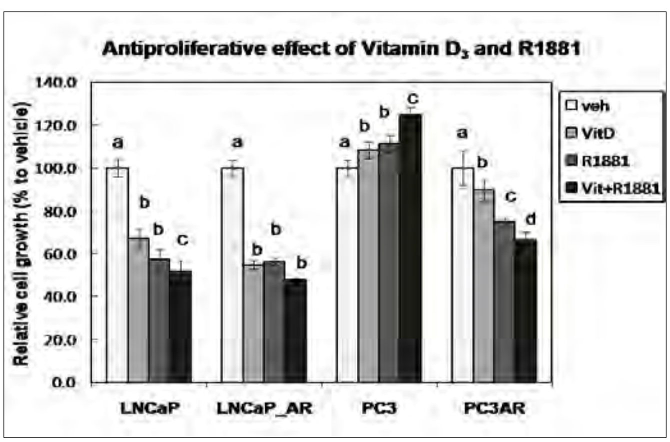


Figure 5 Expression of AR and CYP24A1in androgen-dependent and independent cell lines. Cells were plated in 6 well plate to 60% confluence and treated with 0.1% ethanol, 10nM 1α25(OH)2 D3 and /or 10nM R1881 for 48 hr. Cell lysates were immunoprecipitated with anti CYP24A1and probed for AR,CYP24A1and ß-actin antibody.

C.4. *Androgen Administration Enhances the Growth Inhibitory Effect of Vitamin D_3:* We studied the actions of androgen (alone or together with vitamin D_3) administration on cell using WST-1 colorimetric assay (**Fig.5**). In LNCaP (expresses a mutant AR and displays androgen-regulated growth) and PC3AR cells (stablely transfected expressing the androgen receptor (AR)), R1881 (a synthetic androgen) administration caused 43% decrease in the cell growth. Similarly 1,25$(OH)_2D_3$ simultaneously inhibited LNCaP cell growth by 33%. The combined effect of R1881 and Vitamin D_3 were more pronounced, showing almost a 50% inhibition by72 hr. The synergistic effect of the androgens and Vitamin D_3 is more potent, exhibiting a strong anti proliferative effect in AR-dependent cells within 72 hrs. In contrast, AR-independent PC3 (lacking androgen receptor) was not significantly growth inhibited by R1881 and Vitamin D_3. LNCaP_AR cells (androgen receptor present but growth resistant to androgens) showed a pattern similar to LNCaP. The cell line lacking AR (PC3) was resistant to Vit D3 or R1881 administration and this was reversed when androgen receptors were stably transfected (PC3AR). Studies have shown that DHT has a biphasic effect on LNCaP cells, at low doses (0.1nM- 1 nM), it has a growth stimulatory effect and at higher doses there is a gradual loss of the growth stimulatory action of DHT (20). Our study was done at 100nM R1881, where we see a growth inhibition.

Figure 7 Effect of androgen on the growth inhibitory effect of 1,25$(OH)_2D_3$in LNCaP, LNCaP_AR, PC3 and PC3AR cells. Cells were grown to 50% confluence, treated with 0.1% ethanol, 10nM 1,25$(OH)_2D_3$ and /or 100nM R1881 and harvested after 72 hr. Colorimetric cell proliferation assay by WST-1 reagent was used to measure the cell proliferation (n=6, 2 exp. replications). Two-way ANOVA shows statistically significant interaction ($p<0.05$) between 1,25$(OH)_2D_3$ and R1881 in LNCaP and PC3AR cell lines



C5. *Effect of Synthetic Androgen (R1881) on CYP24A1and Androgen Receptor Gene Expression:* Androgen-dependent and -independent human prostate cancer cell lines (as described above) were pretreated with 10nM 1,25$(OH)_2D_3$in absence or presence of 10nM R1881 and were harvested after 48 hr. As expected (18,21), vitamin D_3 induction significantly enhanced the expression of CYP24A1mRNA with the highest level of induction being found in the LNCaP cell line (**Fig.6**). Similar results were reported in (18,21). In contrast, pre-incubation with 10nM R1881 together with 10nM 1,25$(OH)_2D_3$ significantly suppressed the expression of CYP24, indicating that R1881 at physiological concentration protects Vitamin D_3 from catabolism. In androgen-independent cell lines, PC3, DU145 and LNCaP_AR (C-42, a stable androgen independent cell line), the expression of CYP24A1mRNA induced by 1,25$(OH)_2D_3$ was not significantly inhibited by androgens. Thus, androgens down regulate CYP24A1gene expression in androgen-dependent cell lines, there by enhancing the antiproliferative functions of Vitamin D_3. Western blot analysis demonstrated a robust expression of AR in LNCaP and PC3AR cells (not shown) with no expression in PC3 cells **(Fig.6).** AR was stabilized and showed a graded response with induction of 10nM 1,25$(OH)_2D_3$and 10nM R1881, whereas, in PC3, DU145 and LNCaP_AR cells there was little or no expression of AR. CYP24A1protein is over-expressed in all human prostate cancer cell lines. CYP24A1expression is enhanced with the administration of 10nM 1,25$(OH)_2D_3$ in both AR-dependent (LNCaP) and AR independent (PC3) cell line.

R1881 administration to androgen-responsive prostate cancer cells enhances the antiproliferative activity of Vitamin D_3 (**Fig.8**) and protects Vitamin D_3 from inactivation by suppressing CYP24A1expression. Results suggest that androgen ablation actually converts the prostate cancer cells to a state of vitamin D resistance.

Figure 8 Effect of androgen on the level of 24-hydroxylase mRNA in LNCaP (A), PC-3 AR (B) and PC3 (C) cells. Cells were treated with 0.1% ethanol, 10nM 1,25$(OH)_2D_3$and /or 10nM R1881 for 48 hr. Total RNA was isolated and CYP24A1mRNA estimated by real-time RT PCR (n=3). Two-way ANOVA shows statistically significant interaction between 1α25(OH)2 D3 and R1881 in LNCaP and PC-3 AR cell lines .

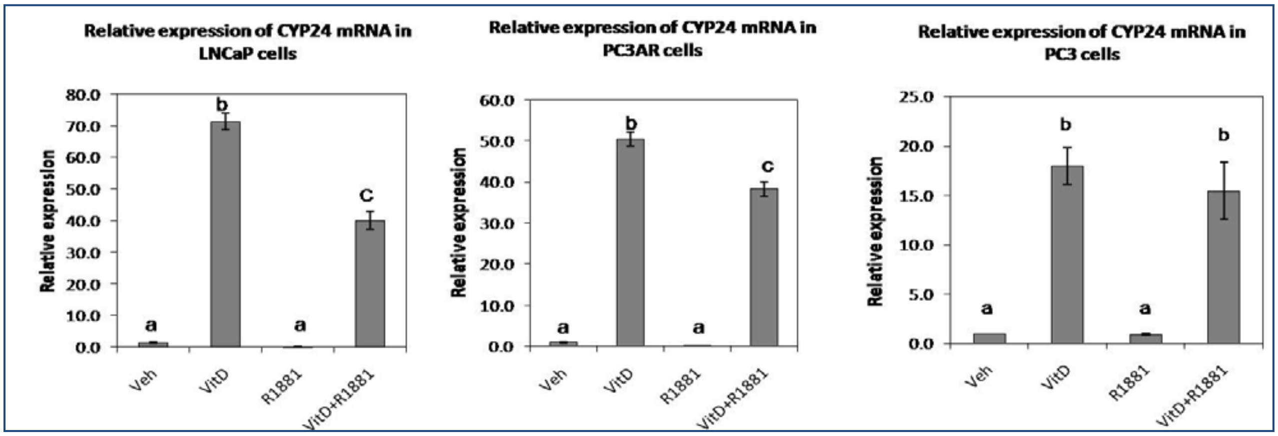
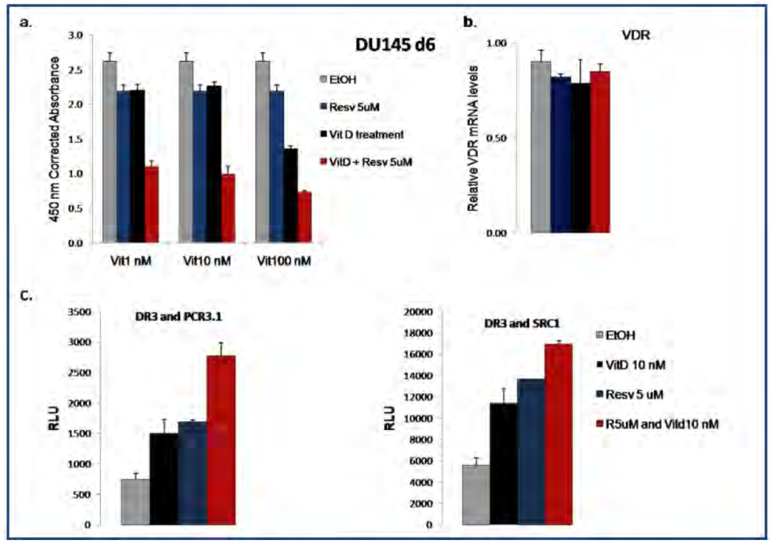


Figure 9 Effect of androgen on the growth inhibitory effect of 1,25$(OH)_2D_3$in LNCaP, LNCaP_AR, PC3 and PC3AR cells. Cells were grown to 50% confluence, treated with 0.1% ethanol, 10nM 1,25$(OH)_2D_3$ and /or 100nM R1881 and harvested after 72 hr. Colorimetric cell proliferation assay by WST-1 reagent was used to measure the cell proliferation (n=6, 2 exp. replications). Two-way ANOVA shows statistically significant interaction ($p \leq 0.05$) between 1,25$(OH)_2D_3$ and R1881 in LNCaP and PC3AR cell lines

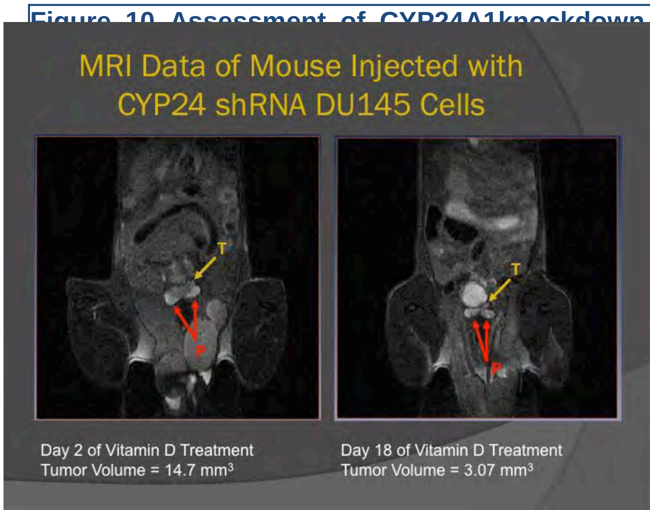


C.5. *Resveratrol Potentiates The Anti-Proliferative Effects of Calcitriol by Enhancing The Transcriptional Activity of the VDR:* Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a polyphenol highly enriched in grapes, berries, peanuts and other dietary sources. Resveratrol exerts inhibitory effects on the initiation, promotion, and progression of carcinogenesis by modulating signal transduction pathways that control cell division and growth, apoptosis, inflammation, angiogenesis, and metastasis (19). Treatment with low-dose resveratrol and calcitriol in combination drastically inhibits cell proliferation. No single concentration was effective (**Fig. 9a**). These agents used alone or in combination; do not induce VDR gene expression (**Fig. 9b**). Potentiation occurs through a VDRE element as shown by luciferase assays (**Fig. 9c**). A synergistic transcriptional effect was obtained when both resveratrol and calcitriol were used on the VDRE. To define the molecular mechanisms underlying this potentiation, we tested whether the interaction of VDR with coactivators is enhanced in presence of both components. One important interacting cofactor with VDR is the p160 family member, SRC-1. Transfection with an SRC-1 expression vector enhanced the transcriptional activity on a VDRE in presence of resveratrol and calcitriol (**Fig. 9d** right panel).

Specific Aim #2 To Enhance Vitamin D inhibition of prostate cancer proliferation by CYP24A1siRNA in vivo.

We generated stable CYP24A1shRNA expressing prostate cancer cell lines (section C.3) and enhanced the anti-proliferative effect of calcitriol when compared to control cells expressing non-targeting shRNA. The control shRNA (a commercially available general purpose 21 mre, 48% GC) has no homology to human, mouse or rat mRNA . SCID mice were maintained on a vitamin D3 deficient diet containing 0.5% calcium (#5826-CI, Purina) for 2 weeks prior to use. The recipient mice were anesthetized and injected intraprostatically with 1 X 106 stably transfected prostate cancer cells. This cell concentration achieves consistent local tumor growth within 7 days of implantation. Two weeks after injection, mice were separated into groups of 8 animals each and treated with control- sesame oil with 2% ethanol or 0.5 µg/kg 1,25(OH)2D3 (in sesame oil with 2% ethanol) every other day by oral gavages with a 20 gauge intragastric feeding tube.

Figure 10. Assessment of CYP24A1knockdown using DU145 cells injected orthotopically in the



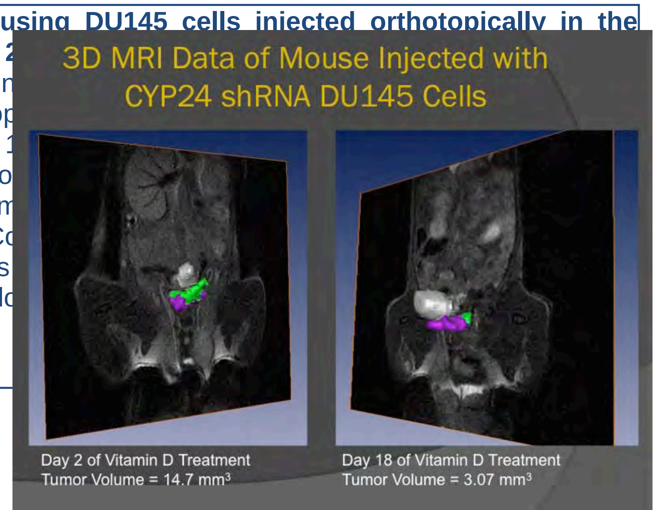
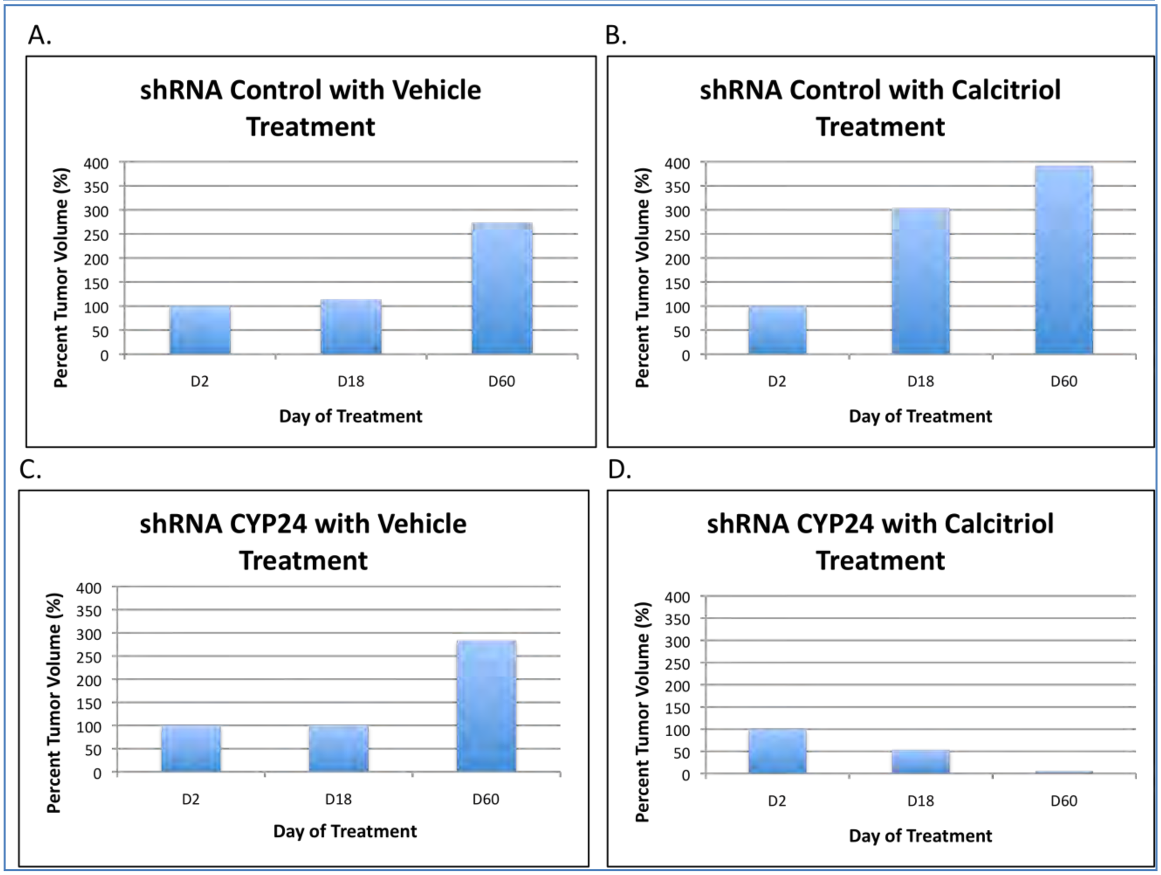


Figure 11: Graphs A-D show tumor volumes over the course of treatment as a percentage of the starting tumor volume at treatment day 2. Each graph shows data from representative treatment groups in this orthotopic xenograft experiment where mice were injected intraprostatically with DU145 prostate cancer cells stably transduced with either CYP24 targeted or control shRNA. The mice were then given treatments every other day with calcitriol or vehicle.



In these studies, MR images were obtained using a Bruker Biospin Pharmascan 7.0T spectrometer (Bruker Biospin, Billerica, MA) in the Mouse Phenotyping Core at Baylor College of Medicine so that we could non-invasively follow xenograft tumor growth over time. The average size of the prostate prior to intraprostatic injection of prostate cancer cells was 24 mm^3. In a representative animal receiving DU145 cells stably transfected with the CYP24A1siRNA (DU41 cells), the combined volume of the prostate gland and tumor is 14.7 mm^3 at D2 and decreased in size to 3.07 mm^3 on D18 (Figure 10). In a representative animal receiving DU145 cells stably transfected with the control vector (DUC cells), the combined volume of the prostate gland and tumor is 21.5 mm^3 at D15 and decreases in size to 20.2 mm^3 on D30 (Not shown). The tumor size in the DUC injected animals has not changed in comparison to the tumor size in the DU41 injected animals. These studies will be carried out longer than 30 days because the tumor burden has not reached 1.5 cm^3. There are a total of 8 animals (out of 40 total in the study; n = 2 mice / treatment group) undergoing repeated MR imaging in-order-to monitor tumor growth. Baseline volume (D0) of the prostate gland was determined in two animals and averaged. Figure 11 simply shows the ongoing results at day 60 of treatment expressed as tumor volume calculated by MRI. The tumors grew at similar rates in the presence or absence of calcitriol with control (scrambled) shRNA and with CYP24 shRNA and vehicle only.

Importantly, the mice with the tumor xenograft with CYP24 knockdown showed highly significant inhibition of tumor growth over the same time period.

These studies, although still ongoing, provide some evidence that CYP24A1knockdown in human prostate cancer cells tested in a xenograft model enhances the growth inhibiting/apoptotic actions of Vitamin D administration in prostate cancer. We will perform histological and phenotypic assays to further define the effect of CYP24A1knockdown on prostate cancer cell growth. Once total tumor burden is 1.5 cm, the mice will be anesthetized and cardiac puncture performed to collect serum. Tumor growth and sites of metastasis especially pulmonary and retroperitoneal metastases, commonly observed in advanced prostate cancer will be assessed. Average tumor size and weight will be measured and tumor volume will be calculated using the equation: tumor volume (cm3) = 0.523 x [length (cm) x width2 (cm2)]. Specimens will be fixed in formalin and embedded in paraffin for hematoxylin-eosin staining or for proliferation markers immunostaining (Proliferating Cell Nuclear Antigen) and apoptosis using the TUNEL assay. Specimens will be cryopreserved for further protein and/or RNA analysis of proliferation and apoptosis markers. Serum collected by cardiac puncture will be used for calcium measurement to detect hypercalcemia (normal calcium values being about 8 mg/dl) and kidney sections will be stained for calcium using the von Kossa stain or Alizarin Red S. We will analyze intra prostatic Vitamin D3 content by HPLC to define the functional effect of CYP24A1knockdown.

Major Findings of Progress Report Studies are:

a) CYP24A1(RNA and Protein) is over-expressed in prostate cancer and increases with TNM

b) CYP24A1siRNA blocks CYP24A1expression and caused vitamin D_3 resistant prostate cancer to become vitamin D_3 sensitive and to once again be growth inhibited by $1,25(OH)_2D_3$.

1. Growth inhibition is seen in vivo and in vitro

c) Androgens enhance the antiproliferative action of vitamin D_3 in prostate cancer by inhibiting CYP24A1expression

d). Resveratrol markedly enhanced vitamin D_3 action working through SRC-1 and VDR

Work Remaining to Be Completed During the No Cost Extension:

a). Aim #1:

1. Analysis of CYP24A1enzymatic activity and assessment of the half-life of $1,25(OH)_2D_3$.

2. Completion of studies of induction of apoptosis by vitamin D

b). Aim #2

1. Completion of the xenograft studies described above

2. Direct delivery of shRNA in vivo through injection directly into the xenograft or into the tail vein.

Two manuscripts are nearing completion for submission: One on CYP24A1levels and effect of knockdown on prostate cancer growth in vivo and in vitro. The second manuscript will focus on the requirement for androgens and the androgen receptor for efficient vitamin D action. A third paper will be prepared in the future on the work to be completed during the no cost extension.